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14. ABSTRACT
Delta-N-p63 plays a critical role in making decision to preserve or forfeit mammary stem cells/progenitor cells' self-renewal capacity. In embryonic stem cells, some transcription factors including oct3/4, nanog, c-myc and Klf-4 are critical to maintain self-renewal and multi-potential stasis. Our study revealed that these key transcription factors also exist in adult mammary stem cells/progenitor cells as well as breast cell lines such as IMEC, MCF-10A, SUM102 and MCF-7 cells. Over-expression of ectopic delta-N-p63 could inhibit the proliferation rate of treated cells, and had diverse regulation effects on transcript level of oct3/4, nanog, c-myc and Klf-4 in infected breast cell lines. Retinoic acid treatment also could slow down the growth of treated beast cells, and change the transcript level of these self-renewal related genes. Both of RA treatment and over-expression of delta-N-p63 could increase mammosphere formation capacities in most breast cell lines including IMEC, SUM102 and MCF-7 cells. The mRNA level of oct3/4 and nanog was detectable in mouse mammary stem cells or progenitor cells enriched subpopulation. Additionally, both oct3/4 and nanog transcript level could be regulated by over-expression or removal of delta-N-p63 in mammary stem cells or progenitors fractions, respectively. In human breast cell lines such as SUM102 cells, over-expression of mouse oct3/4 and nanog could increase the mammosphere numbers significantly. On the other hand, removal of delta-N-p63 in MCF-10A cells could decrease the mammosphere formation capacity dramatically. Taken together, all these findings strongly suggested there might be correlation between delta-N-p63 and ES programming genes including oct3/4, nanog, c-myc and Klf-4 to regulate stem cell self-renewal and sustaining of pluripotency in adult mammary gland.

15. SUBJECT TERMS
Delta-N-p63, retinoic acid, oct3/4, nanog, c-myc, Klf-4, self-renewal, maintenance of pluripotency, breast cancer, IMEC, MCF-10A, SUM102, MCF-7

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Introduction:

Recent studies support a tumor stem cell theory, which holds that the cells in which cancer initiation occurs have either inate or acquired retained proliferative capacity and a prolonged lifespan. Throughout female reproductive life the epithelial portion of the mammary gland undergoes multiple periodic regenerative cycles characterized by cellular proliferation and terminal differentiation (1). Continuous regenerative cycling of the epithelial portion of the mammary gland depends upon the existence and activity of Mammary Stem Cells (MSCs) that retain their proliferative capacity and resist termimal differentiation (2). These features confer a prolonged replicative lifespan indicating that such cells may be capable of accumulating mutations and harboring them from the protective effects of apoptosis. In addition, such mammary progenitor cells may be the sites of breast cancer initiation, which is supported by studies in which mammary tumor stem cells were prospectively identified and shown to be uniquely tumorigenic and able to self-renew (3). These studies suggested that cancer initiation is a condition of unregulated or poorly regulated self-renewal and have focused attention upon potential of genetic pathways that regulate self-renewal. The goal of this research is to identify genes and genetic pathways associated MSC self-renewal and to determine their predictive and/or prognostic value in clinical breast cancer.

It has been widely accepted that stem cell cells exist in adult tissues with regenerative capacity and that they play an important role in maintaining cellular stasis within those tissues. (4). They can be defined as cells capable of asymmetric divisions that give rise to daughter cells that independently retain or forfeit their self-renewing capacity. Currently, embryonic stem (ES) cells have been commonly applied for investigation of regulation mechanism of stem cell self-renewal and differentiation process. Several transcription factors, including Oct3/4, Sox-2 and Nanog could contribute to maintain the pluripotency in ES cells (5-11). In addition, many other genes such as c-myc and Klf-4, frequently upregulated in tumors also have been shown to establish and preserve the ES cell phenotype and the rapid proliferation of ES cells in culture. More importantly, the introduction of these four factors (Oct3/4, Sox-2, c-myc and Klf-4) into mouse embryonic or adult fibroblasts issufficient induce the phenotype change from differentiated cells to stem cell-like ones under ES cell culture conditions. These cells exhibit the morphology and growth properties of ES cells and express ES marker genes (12-14). The discovery of this multi-factor reprogramming set identified as Oct3/4, Sox-2, c-mycm Klf-4, and Nanog, has demonstrated a high degree of cellular plasticity within developmentally committed cells. Our previous research has clearly demonstrated the existence of adult stem cells and progenitor cells in mammary gland. Given the recent interest in the

study of composition and function of reprogramming set in ES cells, a profound question has been raised whether similar programming induction exist in adult mammary stem cells and progenitor cells.

There is abundant evidence that the p53 family member, TP63 plays a critical role in the decision to preserve or forfeit self-renewing capacity in adult epithelial stem cells (15-18). The gene encoding TP63 utilizes proximal and distal promoters to produce Trans-Activating (TA-p63) and N-terminally deleted (△N-p63) isoforms (19). In adult mammary gland, several studies indicate that expression status of TP63 reflects preservation or forfeiture self-renewing ability (20). Mutations of TP63 have been shown to underlie broad spectrum of human syndromes, which have been in common defects in the establishment or cellular stasis of a variety of epithelial and apocrine structures (17, 21). These defects are believed to undermine a genetic program of non-regenerative differentiation that ultimately leads to the depletion of stem cells. Additionally, studies using pan-p63 antibody (4A4) indicate that TP63 predominates in the basal/myoepithelial layer of mammary gland, suggesting that TP63 may play an important role in the preservation of mammary progenitors (22). This is further supported by studies indicating that TP63 is a biomarker of stem cells in corneal keratinocytes and that repression of TP63 expression is correlated with the transition to a transient amplifying cell population and terminal differentiation (23).

In support of the work funded the Department of Defense Breast Cancer Research Program grant number W81-XHW-05-1-0350 we have previously reported that delta-N-p63 co-localizes with another stem cell markers known as nestin, in breast basal epithelial cells (24). Furthermore, immunohistochemical analysis of human breast cancer specimens has indicated that nestin is a selective marker of basal epithelial breast tumors and is regulated by ΔN-p63. Additionally we reported that in the embryonic carcinoma cell line NT2/D1, ectopic ΔN-p63 could disrupt retinoic acid-induced differentiation, thereby preserving expression of nestin. These findings are significant for two reasons. First they identify and validate a molecular marker for the basal epithelial breast cancer sub-type, that is traditionally identified solely by the absence of ER, PR and Her2. Second these findings implicate nestin and ΔN-p63 as potential molecular targets of basal epithelial breast cancer subtypes including BRCA-1 mutation carrying tumors. All of these observations are consistent with the role of ΔN-p63 in self-renewal processes collaborating with other critical self-renewal or regeneration related genes, indicating that these ΔN-p63 and nestin may have high diagnostic and prognostic evaluation values in breast cancer due to their roles in normal mammary gland regeneration.

In addition to our findings concerning ΔN-p63 and nestin and in further support of BCRP grant number

W81-XHW-05-1-0350 we additionally report a regulatory relationship between ΔN-p63 and Oct3/4, Sox-2, c-myc, Klf-4 and Nanog in breast cancer cell lines andprimary mammary epithelia. These studies support the notion that ΔN-p63 is a sufficient to confer stem cell-like features and gene expression profiles upon committed lineages of mammary epithelia. We believe that these results support a model for breast cancer initiation in which ΔN-p63 expression and activity are required for tumorigenicity and self-renewing capacity. What follows is a detailed report of the progress associated with this work:

Body:

Materials and Methods:

Breast cancer cell line and immortalized breast epithelial cells culture: The breast cancer cell line including SUM102, MCF-7 and immortalized breast epithelial cells including IMEC, MCF-10A cells were cultured as described previously. The culture media of MCF-7 cells was DMEM media supplemented with 10% fetal brovine serum; the media of SUM102 cells was Ham's F12 supplemented with 10mM Hepes, 5ug/ml insulin, 1ug/ml hydrocortisone, 10ng/ml EGF; the media of IMEC and MCF-10A cells was MEGM supplemented with bovine pituitary extract (BPE), and IMEC media was puromycin selective (5ug/ml). All cell lines were cultured at 37℃, 5% CO2 incubator. For differentiation induction experiments, all cell lines were treated with 1umol/L all-trans retinoic acid and 0.01% DMSO as vehicle control. For ΔN-p63 overexpression experiments, all cell lines were infected with delta-N-p63 alpha and GFP empty vector adenovirus as vehicle control with ration at 1ul/ml. For ΔN-p63 knock down experiments, IMEC, MCF-10A and SUM102 cells were infected with ΔN-p63-α , TP63 DNA binding domain specific siRNA and GFP empty vector adenovirus as vehicle control with ration at 8ul/ml. For Oct3/4 and Nanog overexpression experiments, mouse Oct3/4 and nanog pcDNA-3.1 expression plasmid were transfected into IMEC, MCF-10A and SUM102 cells mediated with Fugene transfection reagent and the transfection protocol was followed as described in instructions.

Cell counting: All breast cell lines were plated into 6-well plates at 10^5/ml cellular density. The second day, the cells were given retinoic acid treatment (DMSO as vehicle control), ΔN-p63 adenovirus infection (GFP empty vector as vehicle control), ΔN-p63 and DNA-Binding Domain (DBD) specific siRNA adenovirus infection (GFP empty vector as vehicle control), mouse Oct3/4 and nanog plasmid transfection (pcDNA3.1 empty vector as vehicle control), respectively. All the cell samples were harvested post 72 hours treatment and counted directly.

Anti-Oct3/4 and Nanog lentiviral shRNA preparation and gene knock down: Lentiviruses expressing shRNA against human Oct3/4 and nanog were purchased from OpenBiosystems. The shRNA plasmid was transfected into 293 cells for letivirus package. The lentiviruscontaining supernatant was harvested post transfection for 72 hours, and spin down, filtered and stored at 4℃. All breast cells were treated with anti-Oct3/4, nanog and GFP empty vector lentivirusas control vehicle vector for 72 hours with ratio (3:1), respectively. Polybrene was added to lentivirus containing supernatant to increase infection efficiency. The total RNA samples were harvested for quantitative PCR analysis.

Western blotting: Western blotting of TP63 (4A4 antibody), Oct3/4, Nanog and c-myc was

conducted as described previously (24). The total protein samples were harvested with NETN buffer [100mM Tris-HCl(pH7.8), 1mM EDTA, 100mMNaCl and 0.1% Triton-X-100]. The primary antibodies included mouse anti-p63 monoclonal antibody 4A4 (BD Pharmingen), donkey anti-Oct3/4 polyclonal antibody (Santa Cruz Biotechnology), rabbit anti-nanog polyclonal antibody (Abcam) and rabbit anti-c-myc polyclonal antibody (Santa Cruz Biotechnology). All antibodies were diluted according to manufacturer's protocol.

Quantitative PCR and semi-quantitative PCR: Total RNA samples were harvested and 0.2 ug RNA was applied for making cDNA with reverse transcriptase kit (Bio-Rad). All applied genes primers are listed as follows: human ΔN-p63 : sense 5'-ATG TTG TAC CTG GAA AAC-3', antisense 5'-ATG GGG CAT GTC TTT GC-3'; human Oct3/4: sense 5'-ACA TGT GTA AGC TGC GGC C-3', antisense: 5'-GTT GTG CAT AGT CGC TGC TTG-3'; human nanog: sense 5'-CAA AGC CAA ACA ACC CAC TT-3', antisense 5'-TCT GCT GGA GGC TGA GGT AT-3'; human c-myc: sense 5'-GCG TCC TGG GAA GGG AGA TCC GGA G-3', antisense 5'-TTG AGG GGC ATC GTC GCG GGA GGC T-3'; human Klf-4: sense 5'-CCC ACA CAG GTG AGA AAC CT-3', antisense 5'-ATG TGT AAG GCG AGG TGG TC-3'; human SOX-2: sense 5'-CGT GAG TGT GGA TGG GAT TGG-3', antisense 5'-GG AAA TGGGAG GGG TGC AAA AGA G-3', human GAPDH: sense 5' GCT TGT CAT CAA TGG AAA TCC C-3', antisense 5'-TTC ACA CCC ATG ACG AAC ATG-3'; mouse Oct3/4: sense 5'-ATT GAG AAC CGT GTG AGG TGG AGT-3', antisense 5'-TGG CGA TGT GAG TGA TCT GCT GTA-3'; mouse nanog: sense 5'-AAG TAC CTC AGC CTC CAG CA-3', antisense 5'-CGT AAG GCT GCA GAA AGT GC-3'; mouse c-myc: sense 5'-CAC CAT GCC CCT CAA CGT GAA CTT CA-3', antisense 5'-TTA TGC ACC AGA GTT TCG AAG GTG TT-3'; mouse Klf-4: sense 5'-CAC CAT GGC TGT CAG CGA CGC TCT GC-3', antisense 5'-ACA TCC ACT ACG TCG GAT TTA AAA-3'; mouse SOX-2: sense 5'-AGA ACC CCA AGA TGC ACA AC-3', antisense 5'-ATG TAG GTC TGC GAG CTG GT-3'; mouse GAPDH: sense 5'-GAA GAC ACC AGT AGA CTC CAC GAC A-3', antisense 5'-ATG TTC CAG TAT GAC TCC ACT CAC G-3'. For quantitative PCR, the reaction system was: 12.5ul Sybr-green Supermix reagent (Bio-Rad), 0.5ul sense primers (10umol/L), 0.5ul antisense primer (10umol/L), 0.5ul cDNA, 11.0ul water. For semi-quantitative PCR, the reaction system was: 2.5ul PCR buffer (10 ×), 1.0 ul MgCL2 (2.5mmol/L), 0.5ul dNTPs (10mmol/L), 0.5ul sense primer (10umol/L), 0.5ul antisense primer (10umol/L), 0.2ul Tag polymerase (Invitrogen), 0.5ul cDNA, 19.3ul water. The PCR program was 94℃ for 10 min, then 94℃ 30s, annealing temperature (human nanog, human Klf-4, mouse Klf-4, mouse SOX-2 primers were 57℃, mouse nanog, mouse GAPDH primers were 58℃, human Oct3/4, human GAPDH primers were 54℃, human SOX-2, mouse c-myc primers were 62℃, human SOX-2 primer was 62℃, human c-myc primer was 68℃ and human delta-N-p63 was 50℃, respectively) for 45s, 72℃ for 1min, for 40 cycles, 72℃ for 10min, then kept at 4℃ forever. The quantitative PCR was analyzed with Bio-Rad Q-PCR OPTICON software, and semi-quantitative PCR

was run by gel electrophoresis.

Mammosphere formation assay: All the breast cell line samples were harvested after 72 hours treatment (Retinoic acid, delta-N-p63 over-expression, TP63 knock down, Oct3/4 and nanog transfection and letivirus knock down, respectively). The treated cells were plated on 24-well low-binding plates at 5×10^4/well cellular density. Primary mammosphere were formed in 10 days to 2 weeks. The mammosphere structure were collected and digested with 0.25% Trypsin and EDTA for 10 min, and re-plated on 24-well low-binding plates for secondary mammosphere formation assay.

Mice maintenance, identification of breast stem cell, progenitors enriched mouse cellular population: B6/129 wild type and PATCH (-/+) heterozygous mice (Jackson Laboratories) were bred and maintained according to institutional guidelines. Experimental protocols were approved by the IACUC at Dartmouth Medical School. The protocol of isolation of mammary stem cells, progenitors was described previously (25).

Results:

Retinoic acid treatment and ecotopic ΔN-p63 decrease the growth rate of breast epithelial cell lines.

The treatment of retinoic acid could inhibit the proliferation of immortalized basal epithelial cells (IMEC), and cause IMEC cells to start differentiation. Removal of retinoic acid was insufficient to restore cell proliferation, indicating that such differentiation-inducing effect was irreversible. Multi-potential progenitor cells tend to have prolonged life-span and self-renewal capacity, characterized by the ability to resist differentiation and re-enter into proliferation cellular phase. There is abundant evidence that ΔN-p63 expression and acitivity play a key role in progenitor cells' proliferation or differentiation decision-making process. We have detected expression of ΔN-p63 in SUM102 and MCF-7 breast cancer cell lines. MCF-7 cells are a luminal epithelaial breast cancer cell line characterized by estrogen receptor and cytokeratin 19 expression, which is inconsistent with the basal epithelial phenotype of ΔN-p63-positive cells. Immunofluorescence studies detected strong nuclear expression of ΔN-p63 in a very small subpopulation which we estimate to be less than .01% of MCF-7 cells. This finding likely accounts for the observation that ΔN-p63 could be detected by RT-PCR but not be western blot in MCF7 cells. This observation coupled to signature distribution of ΔN-p63 in normal breast basal epithelial cells and basal/myoepithelial breast cancer subtype, suggested that ΔN-p63 might be also essential to breast cancer stem cell or progenitor cell self-renewal. To further study the biological function of ΔN-p63 in breast epithelial cells, ΔN-p63 was overexpressed by infection breast cell lines including IMEC, MCF-10A, SUM102 and MCF-7 cells, with adenoviral-ΔN-p63-α- IRES -GFP (adeno-ΔN-p63-α) or adeno-GFP (control). Western

blotting and quantitative PCR confirmed ΔN-p63-α expression. Cell counting indicated that ectopic delta-N-p63 could decrease the proliferation rate in all infected breast epithelial cells significantly. Similarly, these breast epithelial cell lines were treated with 1umol/L all-trans retinoic acid for 72 hours, and all the samples were underwent cell counting directly. Compared with DMSO vehicle control group, RA treatment could also inhibit the growth of breast cell line significantly.

Retinoic acid treatment has variable influences on transcript levels of Oct3/4, nanog, c-myc, Klf-4 and SOX-2 in breast cell lines.

Our studies have shown that retinoic acid could down-regulate expression of ΔN-p63 in immortalized basal epithelia cells (IMEC), and loss of ΔN-p63 could lead up to forfeiture of self-renewal as well as irreversible cellular differentiation. In embryonic carcinoma NT2/D1 cells, ectopic ΔN-p63 could block retinoic acid induced differentiation and preserved the expression of genes down regulated by retinoic acid. All these findings suggest ΔN-p63 could prevent progenitor cells from retinoic acid induced differentiation to keep cellular self-renewal ability. IMEC, MCF-10A, SUM102 and MCF-7 cells ere treated with 1umol/L retinoic acid and 0.01% DMSO as vehicle control. Total RNA samples were collected at 72 hours after RA or DMSO treatment. Then, quantitative and semi-quantitative PCR were used to detect the transcript level of Oct3/4, nanog, c-myc, Klf-4, and SOX-2 post retinoic acid treatments. The transcripts of Oct3/4, nanog, c-myc, Klf-4 could be clearly detected in IMEC, MCF-10A, SUM102 and MCF-7 cells, but the transcript level of SOX-2 in breast epithelia cells was not detectable. These observations suggested the existence of genes key to maintaining the pluripotency of embryonic stem cells in mammary epithelial cells and breast tumor cells, but their integrated compositions and functions could be different in adult mammary organ. Semi-quantitative data clearly showed the transcript levels of ΔN-p63 , Oct3/4, nanog, c-myc, Klf-4 decreased in MCF-10A cells post RA treatment, respectively. Retinoic acid could also down-regulate ΔN-p63 transcript level in IMEC cells, consistent with our previous study. Interestingly, the mRNA level of other reprogramming genes such as Oct3/4, nanog, c-myc and Klf4 was up regulated by retinoic acid in IMEC cells. In breast cancer cell lines, SUM102 and MCF-7 cells, retinoic acid treatment for 72 hours could significantly increase transcript level of ΔN-p63 , Oct3/4 and Nanog. In addition, up-regulation of transcript level of c-myc and Klf-4 could also be detected in SUM102 cells, while c-myc and Klf-4 mRNA was decreased with RA treatment in MCF-7 cells.

Ectopic ΔN-p63-α has diverse effects on transcript level of Oct3/4, Nanog, c-myc, Klf-4 and SOX-2 in breast cell lines.

Our previous study has demonstrated that ectopic ΔN-p63 could inhibit the proliferation of most

malignant or immortalized breast epithelial cells. In NT2/D1 cells, ectopic ΔN-p63 also could block RA-induced differentiation by preserving transcript level of nestin, a stem cell and regeneration biomarker. Taken together, these observations suggest that ΔN-p63 has some influence on message level of genes correlating to maintenance of self-renewal capacity and pluripotency of stem cell and progenitors. We tested the effects of ectopic ΔN-p63 on some candidate genes including Oct3/4, Nanog, c-myc, Klf-4 and SOX-2 with semi-quantitative and quantitative PCR in breast cell lines such as IMEC, MCF-10A, SUM102 and MCF-7 cells. RT-PCR data indicates that over-expression of ΔN-p63-α in IMEC cells could up-regulate the transcript level of Oct3/4, Nanog, c-myc, but down-regulate message level of Klf-4. In terms of MCF-10A cells, another immortalized basal breast epithelia cells, ectopic ΔN-p63-α could increase transcript level of Oct3/4, c-myc and Klf-4, but decrease mRNA level of nanong. In malignant breast cell lines, SUM102 and MCF-7 cells, although their genetic profiling and epithelial phenotype are different, ectopic ΔN-p63-α had similar positively regulated effects on the transcript level of Oct3/4, c-myc and Klf-4. With respect to Nanog, its transcript level was down-regulated in SUM102 cells, but up regulated in MCF-7 cells.

ΔN-p63 ablation in IMEC, MCF-10A and SUM102 alters the transcript levels of Oct3/4, Nanog, c-myc and Klf-4.

Our investigation had explicitly revealed the biological influences of ectopic ΔN-p63-α on the transcript level of reprogramming genes such as Oct3/4, Nanog, c-myc and Klf-4 in immortalized and transformed breast epithelial cells. To further investigate the biological role of ΔN-p63-α in stem cells and progenitors self-renewal regulation process, loss of ΔN-p63-α function experiments of were conducted in IMEC, MCF-10A and SUM102 cells, all of which having detectable transcript and protein level of ΔN-p63-α . Although there is a small sub-population cells that positively with ΔN-p63 (4A4 antibody) in MCF-7 cells, it was not included in such ΔN-p63 knock down experiment due to lower endogenous ΔN-p63 expression level. Adenoviruses expressing shRNAs against the TP63 DNA-binding domain (DBD) and the α-specific C-terminus (α-specific) were co-infected to ensure ΔN-p63-α knock down efficiency. At 24 hours post-infection all breast cell samples were harvested for cell counting directly. Total RNA was collected for making cDNA and further quantitative and semi-quantitative PCR analysis. The cell counting data did not detect any significant changes of proliferation rates of all three breast cells, suggesting removal of ΔN-p63 in IMEC, MCF-10A and SUM102 cells could not result in growth arrest by itself. Retinoic acid treatment could cause both inhibition of grow rate in all three breast cell lines, lower message levels of ΔN-p63 in IMEC and MCF-10A cells, and up-regulation of ΔN-p63 transcript level in SUM102 cells. Taken together, these data suggest that RA-induced cellular proliferation inhibition be not the direct result of transcript level

change of ΔN-p63 in these breast cell lines.

In terms of transcript levels of Oct3/4, Nanog, c-myc and Klf-4, ΔN-p63 ablation of ΔN-p63-α led up to different effects. In IMEC cells, removal of ΔN-p63 could down regulate transcript level of Oct3/4, Nanog, up regulate message level of Klf-4, had no dramatic effects on mRNA level of c-myc. In MCF-10A cells, knock down ΔN-p63 was showed to decrease transcript level of Oct3/4, Nanog, but no significant influence on message level of c-myc and Klf-4. In basal epithelial breast cancer cell line SUM102 cells, the decrease of ΔN-p63 expression level could also cause down-regulations of transcript level of all four genes including Oct3/4, Nanog, c-myc and Klf-4. All these findings implied the role of ΔN-p63 played within integrated net-work of stem cells and progenitor cells self-renewal and proliferation regulation process.

Over-expression of mouse Oct3/4 and Nanog in human breast cell lines had no significant effects on their proliferations and endogenous ΔN-p63 transcript level.

Our investigation had successfully revealed the role of ΔN-p63 involved in the regulation of self-renewal and maintenance of multi-potential phenotypes. To further study the regulation pathways involved ΔN-p63 and Oct3/4 and Nanog transfection experiments were applied with pcDNA3.1 eukaryotic expression plasmid. IMEC, MCF-10A and SUM102 cells were transfected with mouse Oct3/4 and Nanog expression plasmids, respectively. After transfection for 72 hours, all transfected cell samples were harvested for cell counting directly, and total RNA was also collected for PCR assay. Similar to ΔN-p63 knock down experiments, over-expression of mouse Oct3/4 and Nanog in human breast cell line had only subtle effects on cellular growth rates based on cell counting data. Further semi-quantitative PCR analysis revealed that there was no significant change of ΔN-p63 transcript level in Oct3/4 or Nanog over-expressed breast cells. These studies indicated that while ΔN-p63 has clear effects on expression of Oct3/4 and Nanog, these effects were not reciprocal. This observation supports a model in which ΔN-p63 exerts control over the stem-cell phenotype by regulating expression of components of the reprogramming gene set.

Ablation human Oct3/4 and Nanog with lentivirus expressing shRNA against Oct3/4 and Nanog in breast cell lines.

Lentiviruses expressing shRNA against human Oct3/4 and Nanog were purchased from OpenBiosystems. Each gene specific shRNA was composed of five different shRNA sequences respectively. Infected cells were harvested for cell counting after infection with shRNA letivirus for 72 hours, and total RNA was also collected for semi-quantitative and quantitative PCR analysis. Our PCR data clearly showed that both human Oct3/4 and Nanog genes could be knock down with some

shRNA expressing letiviruses efficiently. Cell counting data also did not demonstrate any dramatic change of proliferation rate of Oct3/4 or Nanog knocked down breast cells. In addition, semi-quantitative RCR results did not show significant change of transcript level of delta-N-p63 in these infected breast cells.

Over-expression ΔN-p63, mouse Oct3/4 and Nanog, knock down of human delta-N-p63 could dramatically influence the formation of mammoshpere in breast cells.

Mammoshpere-forming capacity is the ability of mammary epithelial cells to grow in an attachment-independent manner assay and has been widely used as a read-out for stem features, while formation of secondary mammospheres has been reported as evidence of self-renewing capacity. Both stem cells and progenitor cells can form primary mammospheres, but only stem cells are able to form secondary mammospheres. Our assay evidently demonstrated that all the four breast cell lines including IMEC, MCF-10A, SUM102 and MCF-7 cells could form both primary mammosphere and secondary mammoshpere, further implicating existence of both stem cell-like and progenitor cell-like cells in these cell lines, along with cellular heterogeneity in tumor mass. We further investigated the influence of over-expression of ΔN-p63 ,Oct3/4 and Nanog on mammosphere-formation capacity in breast cell lines. Our data demonstrated that ectopic ΔN-p63-α , Oct3/4 and Nanog could dramatically increase the secondary mammosphere formation in SUM102 cells. Over-expression of ΔN-p63-α in IMEC cells, MCF-7 and MCF-10A cells also could induce more secondary mammosphere formation. Conversely, ablation of ΔN-p63-α with shRNA resulted in a significant decrease of secondary mammosphere formation in MCF-10A cells. All these findings strongly support the role of ΔN-p63 , Oct3/4 and Nanog in mammosphere formation process in mammary cell lines.

Retinoic acid exerts diverse effects on mammosphere formation capacity of breast cell lines.

Our study had clearly revealed the proliferation inhibitory effects of retinoic acid on breast cell lines including IMEC, MCF-10A, SUM102 and MCF-7 cells. In addition, retinoic acid could regulate the transcript level of Oct3/4, Nanog, c-myc, Klf-4 genes in breast cell lines, which were key to maintain the self-renewal capacity and pluripotency of ES cells. To test the biological effects of RA on mammosphere formation capacities of breast cell lines, IMEC, MCF-10A, SUM102 and MCF-7 cells were treated with 1μM all-trans retinoic acid and 0.01% DMSO as vehicle control. At 72 hours post treatment, all treated cells were collected and re-plated into 24-well low-binding plates at 5×10^4/well

cellular density. Mammosphere quantification clearly showed that retinoic acid could increase the primary mammoshpere numbers in IMEC, SUM102 and MCF-7 cells, but decrease the mammosphere formation ability of MCF-10A cells. Primary mammosphere structures were harvested and digested into individual cells with 0.25% trypsin and EDTA, for secondary mammosphere formation assay. In IMEC and SUM102 cells, secondary mammosphere numbers in RA pre-treated group were detected higher than DMSO pre-treated cells. However, secondary mammosphere formation ability was decreased in RA pre-treated ones. These findings, together with transcript level change of self-renewal and pluripotency maintaining genes post RA treatment, strongly indicate that retinoic acid can regulate the mammosphere formation ability in breast cells through regulation of expression of reprogramming genes including Oct3/4, Nanog, c-myc, Klf-4 and ΔN-p63. This finding suggests that while retinoic acid is generally considered to be a differentiation agent, it may have a proliferative effect on cells with self-renewing capacity. This observation is also consistent with publications from our lab indicating another morphogen, hedgehog is mitogenic is mammary stem cells and anti-mitogenic in proliferating cells (25). Further study will be necessary to prove this point however it does suggest that differentiating agents may be ineffective at neutralizing tumor stem cells.

Detection and regulation by ΔN-p63 of Oct3/4 and Nanog in enriched fractions of mouse mammary stem cells and progenitor cells.

Mouse mammary stem cells and progenitor cells enriched population were isolated according to protocol described previously. Cellular membrane protein, CD24 and CD29, were applied for distinguish stem cells, progenitor cells and terminally differentiated cells, respectively. Our previous study had clearly displayed the expression segregation of ΔN-p63 and TA-p63 in stem cells and progenitor cells subpopulation. To further discover the distribution pattern of Oct3/4 and Nanog in mouse mammary epithelial subpopulations, mouse specific Oct3/4 and Nanog primers were designed for PCR analysis for mRNA detection. Semi-quantitative PCR data explicitly showed that Oct3/4 transcript could be detected in both stem cell and progenitor cells, and the level in stem cells was higher than progenitor cells in wild-type B6/129 mice. Previously we have shown that activation of hedgehog signaling via Patched1 (Ptch1) heterozygosity resulted in a quiescence defect among mammary stem cells. In $Ptch1^{-/+}$ mammary epithelial subpopulations, there was higher level of Oct3/4 transcript in both stem cells and progenitor cells, respectively suggesting that these transcripts are likely to be up-regulated in activated mammary stem cells. Mouse Nanog transcripts were also detected in both stem cells and progenitor cells subpopulations of wild type and $Ptch1^{-/+}$ mice, respectively. To further investigate the regulation effects of ΔN-p63 on Oct3/4, Nanog, ΔN-p63-α was ablated with adenoviral shRNA expression in progenitor cells and stem cells from wide-type and $Ptch1^{-/+}$ mice, respectively. Semi-quantitative PCR data demonstrated that ablation of ΔN-p63 in

mammary stem cells enriched subpopulations could down-regulate transcript level of Oct3/4 in both wild type and Ptch1$^{-/+}$ mice, while ectopic ΔN-p63-α in progenitor cells enriched subpopulation could up-regulate its mRNA level accordingly. With respect to mice Nanog transcript level, it could be decreased in progenitor cells with ectopic ΔN-p63 , and increased in stem cells with endogenous ΔN-p63 knock down treatment, in both wild type and Ptch1$^{-/+}$ mice, respectively. All of these findings, coupled to transcript level changes of Oct3/4 and Nanog post over-expression and knock down treatment of ΔN-p63 , further suggested the key role of ΔN-p63 in regulation of transcript level of reprogramming genes and cellular self-renewal process in adult mammary gland.

Retinoic acid and ΔN-p63 have diverse effects on the mammosphere formation abilities of mouse mammary epithelial subpopulations.

Mammosphere assay was used to further investigate the biological function of retinoic acid and ΔN-p63 in mammary stem cells and progenitor cells. Preliminary data of primary mammosphere assay revealed that ectopic ΔN-p63 in progenitor cell enriched subpopulation could enhance mammosphere formation. Consistently, knock down ΔN-p63 with shRNA-expressing adenoviruses in stem cell enriched subpopulation could also decrease cellular primary mammosphere formation ability. On the other hand, retionic acid treatment was not observed to have significant effects on mammosphere formation ability of mouse mammary stem cells. Further studies will be necessary to confirm the influences of retinoic acid and delta-N-p63 on mammosphere formation capacities of mouse epithelial subpopulations.

Future directions:

The aim of our research is to identify genes events with clinical diagnostic and prognostic evaluation significance. This aim was initially satisified by the idenficiation of Nestin as a marker of the mammary regenerative compartment and the demonstration that nestin is a selective marker of the basal epithelial breast cancer subtype. This finding coupled to data indicating that this subtype has a poor prognosis and is highly aggressive implies that our identification of nestin will serve as a marker of both predictive and prognostic significance. Our previous study had clearly showed that TP63 is more essential than other p53 family members in such biomarkers localization process in breast cancer due to the definitive role of p63, especially ΔN-p63 in normal breast epithelial regenerative compartment and basal/myoepithelial breast cancer subtype. Abundant evidences suggested ΔN-p63 might play a role in the self-renewal and maintenance of multi-potential stasis of stem cells and progenitor cells in embryonic or adult tissues, collaborating with other critical regeneration related genes. Oct3/4, Nanog, c-myc, Klf-4 and SOX-2, which are essential to maintain stem cells stasis in ES cells, have been reported to induce the differentiated embryonic and adult

fibroblast into stem cell like cells displaying self-renewal and pluripotency phenotype, under ES culture conditions. Given the recent interest in the multiple uses of embryonic and adult stem cells for basic and applied research, attempt have been widely made to explore of the complex transcription factors network maintaining self-renewal and pluripotency in ES and adult stem cells. Our previous study of discover the distribution pattern and biological function of delta-N-p63 in mammary gland and breast cell lines, along with establishment of protocol to identification of stem cells, progenitor cells and terminally differentiated cells enriched populations from mouse mammary epithelial cells, have evidently facilitated the more systematic investigation of regulation mechanism of ΔN-p63 and retinoic acid of cellular self-renewal and multi-potential maintenance process. Our study had successfully prove the existence of transcription factors key to maintain stem cells and progenitor cells stable status, including Oct3/4, Nanog, c-myc and Klf-4 in not only mouse mammary gland, but also multiple breast cell lines such as IMEC and MCF-10A cells, as well as malignant breast cell lines such as SUM102 and MCF-7 cells. In addition, our previous study also revealed the existence of ΔN-p63 transcript and protein in breast regenerative compartment basal epithelial cells, mouse stem cells enriched epithelial subpopulation and same breast cell lines. Together, these findings suggest a correlation between ΔN-p63 and reprogramming genes in regulation process of self-renewal and maintenance of pluripotency in adult mammary gland. They also suggest that within committed lineages, there exists a degree of plasticity that is regulated by ΔN-p63 and other reprogramming genes. Our current investigation system focuses on the networking including ΔN-p63 and other key stem cells and progenitors related genes including Oct3/4, Nanog, c-myc and Klf-4 in regulation of self-renewal and multi-potential maintenance in mammary gland. Because of the role of ΔN-p63 in basal epithelial cells and basal/myoepithelial cells, these critical stem cell programming genes could be potential candidates genes with profound clinical diagnostic and prognostic evaluation significance on the conditions that their correlations with ΔN-p63 in terms of biological function and co-localization could be discovered thoroughly and convincingly.

Our data have clearly showed that both delta-N-63 and retinoic acid have diverse biological influences on proliferation rate, mammoshpere formation abilities and transcript level of essential transcription factors in ES cells and progenitors including Oct3/4, Nanog, c-myc and Klf-4 in either breast cell lines or mouse mammary stem cells and progenitor cells enriched subpopulations. These discoveries further implies there should be collaboration between delta-N-p63 and other critical genes in ES stasis maintenance to co-regulate mammary stem cells and progenitor cells' self-renewal and pluripotency process. Detection of protein level of these stem cell programming genes in mammary gland and breast cell lines would be applied to further verify the discoveries from quantitative and semi-quantitative PCR analysis. Preliminary data of western blotting had clearly showed the

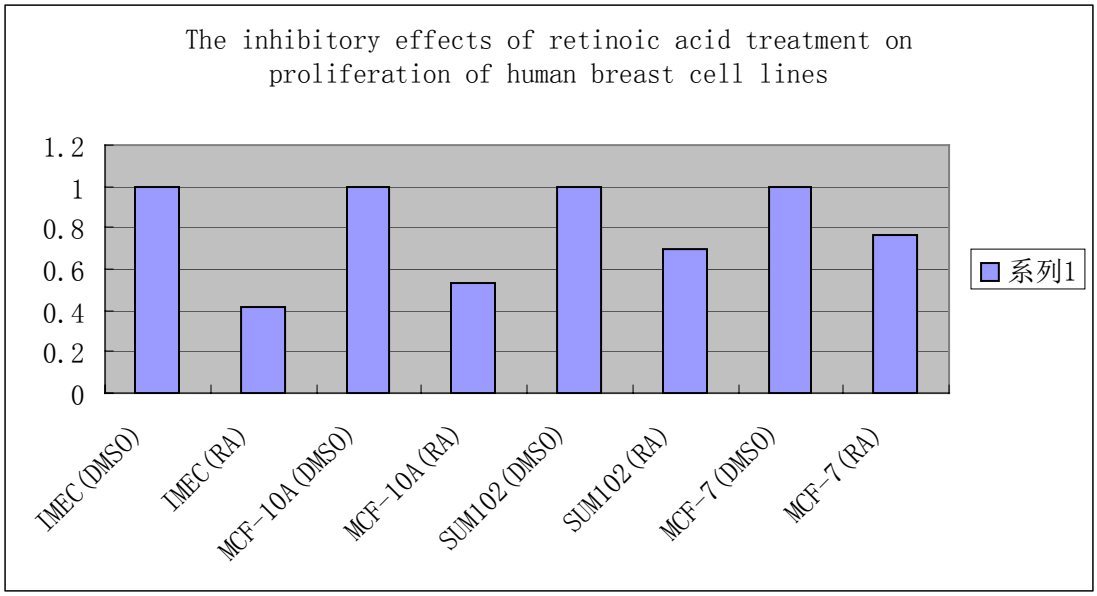
existence of protein Nanog and c-myc in breast cell lines including IMEC, MCF-10A, SUM102 and MCF-7 cells. The protein detection assays of other genes such as Oct3/4, Klf-4 was needed to further prove their functions in mammary gland and breast cell lines accordingly. In addition, immonohistochemistry and immunofluorescence is also necessary in normal breast tissue and various human breast cancer subtypes to further discover clinical significance of these transcription factors key to stem cells and progenitors regulation.

Additionally, loss function and gain function assays of ΔN-p63 , Oct3/4 and Nanog would be performed to study their biological role in mammary stem cells and progenitor cells self-renewal and multi-potential maintenance process, especially in mouse stem cells and progenitors enriched epithelial subpopulations. To further confirm the findings obtained in vitro, ex vivo transplantation experiments recipient mice could also be included. Although there was no mouse work approved in our proposal, more in vivo work should be extremely helpful to determine the biological function of ΔN-p63 and other stem cell programming transcription factors in keeping the stasis of adult mammary stem cells and progenitor cells.

Supporting data:

Figure one: Both retionic acid treatment and ectopic ΔN-p63 could decrease the growth rate of breast cell lines including IMEC, MCF-10A, SUM102 and MCF-7 cells.

All the breast cell lines were treated with 1umol/L retinoic acid and 0.01% DMSO as vehicle control, and delta-N-p63 alpha and GFP empty vector adenoviruses as vehicle control, respectively. After RA treatment or delta-N-p63 infection for 72 hours, all breast cell samples were harvested for cell counting directly.



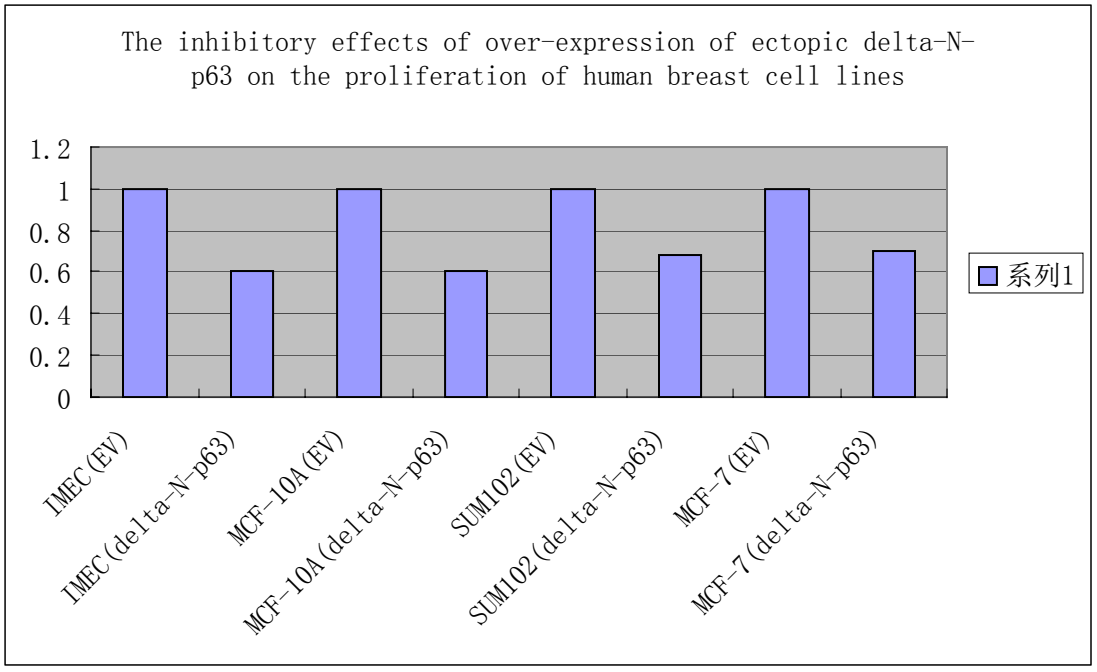


Figure two: The retinoic acid treatment had different influences on transcript level of Oct3/4, nanog, c-myc and Klf-4 in breast cell lines including IMEC, MCF-10A, SUM102 and MCF-7 cells

All of the breast cell lines were treated with 1umol/L retinoic acid and 0.01% DMSO as vehicle control. After RA treatment for 72 hours, total RNA from all breast cell samples were harvested with RNA isolation kit. 0.2 ug RNA for each sample was used to making cDNA and quantitative or semi-quantitative PCR analysis.

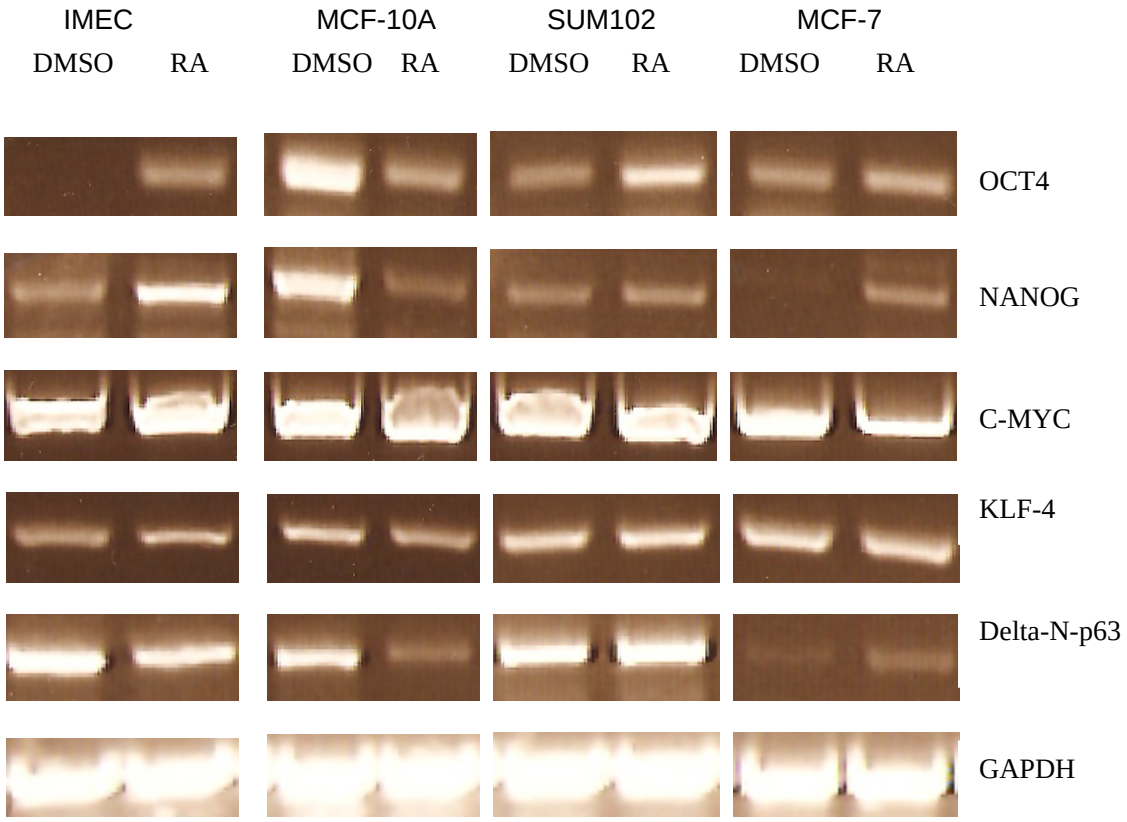


Figure three: over-expression of ectopic ΔN-p63 had diverse effects on transcription level of oct3/4, nanog, c-myc and Klf-4 in breast cell lines including IMEC, MCF-10A, SUM102 and MCF-7 cells.

All of the breast cell lines were infected with 1ul/ml delta-N-p63 alpha and GFP empty adenoviruses as vehicle control. After delta-N-p63 infection for 72 hours, total RNA from all breast cell samples were harvested with RNA isolation kit. 0.2 ug RNA for each sample was used to making cDNA and quantitative or semi-quantitative PCR analysis.

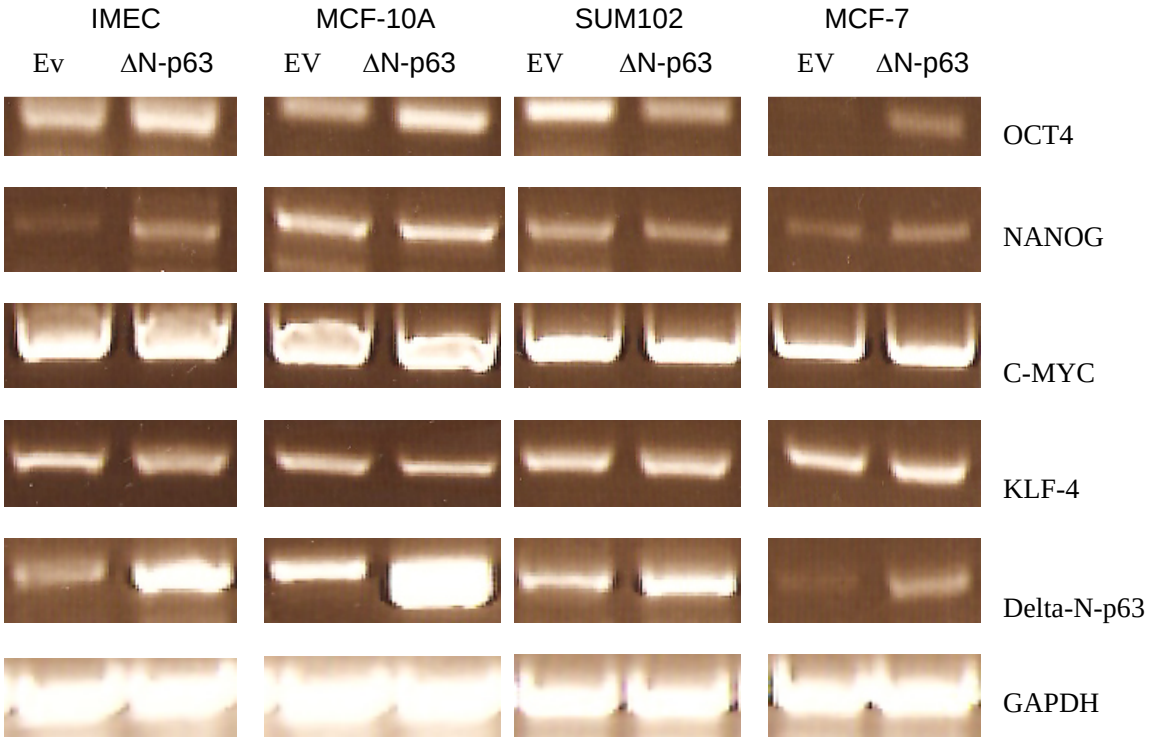
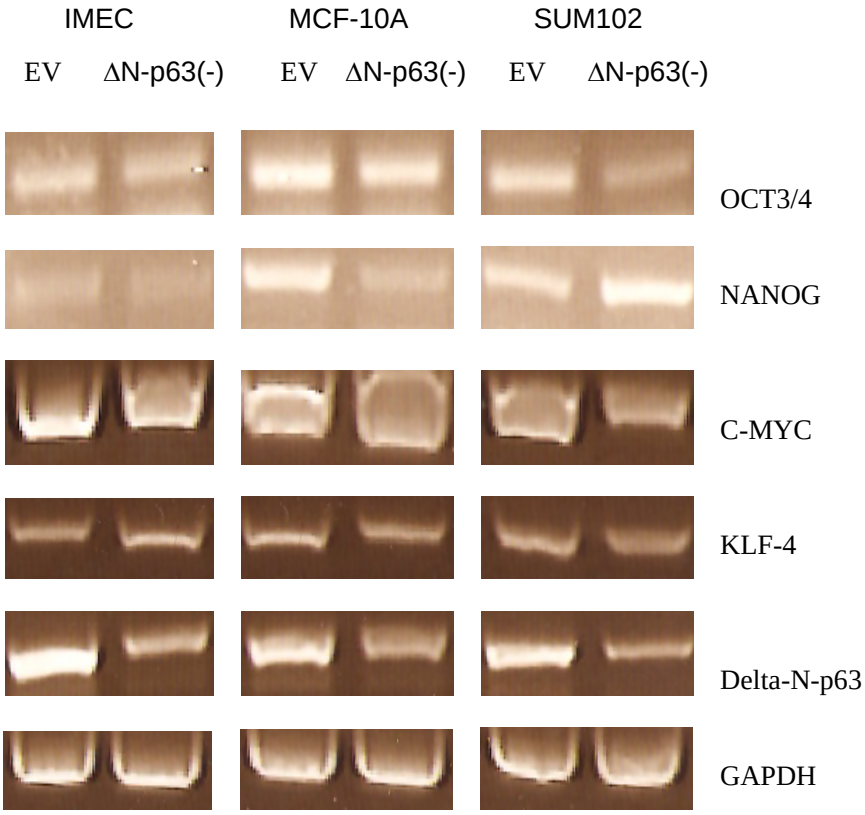


Figure four: ΔN-p63 knocking down in IMEC, MCF-10A and SUM102 cells had no dramatic influences on proliferation rates of these breast cell liens, but could change transcript level of oct3/4, nanog, c-myc and Klf-4 more significantly.

All of the breast cell lines were infected with 8ul/ml adenoviruses expressing siRNA against ΔN-p63 alpha or TP63 DNA binding domain, and GFP empty adenoviruses as vehicle control. After adenoviruses infection for 72 hours, all infected cell were harvested for cell counting directly, and the total RNA from all breast cell samples were harvested with RNA isolation kit. 0.2 ug RNA for each sample was used to making cDNA and quantitative or semi-quantitative PCR analysis.



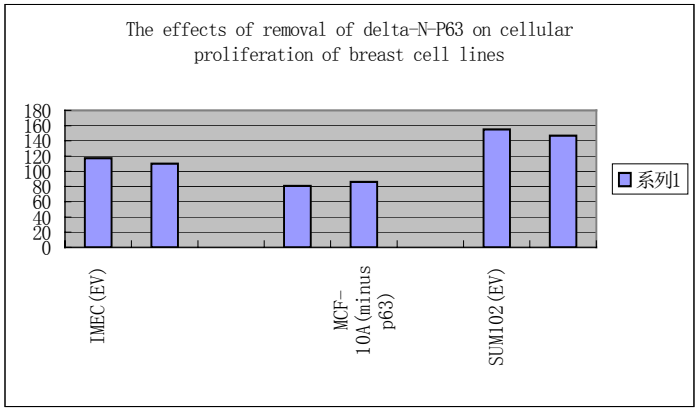
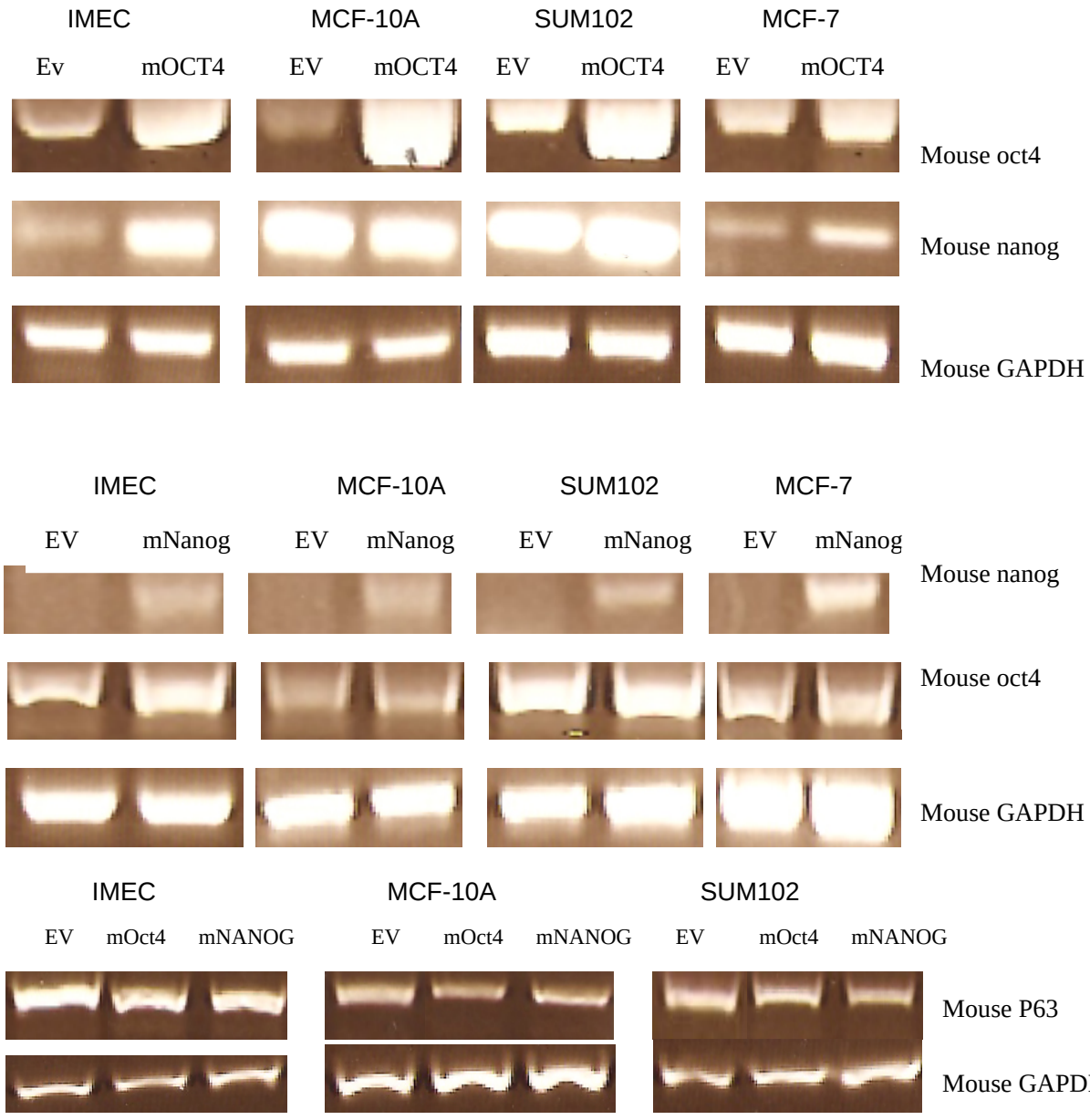


Figure five: overexpression of mouse oct3/4 and nanog in human breast cell lines had no significant effects on their proliferations and endogenous delta-N-p63 transcript level.

The transfection experiments of mouse oct3/4 and nanog into human breast cell lines were mediated with pcDNA3.1 eukaryotic expression plasmid. IMEC, MCF-10A and SUM102 cells were transfected with mouse oct3/4 and nanog pcDNA3.1 plasmid, respectively. After transfection for 72 hours, all transfected cell samples were harvested for cell counting directly, and total RNA was also collected for PCR assay.



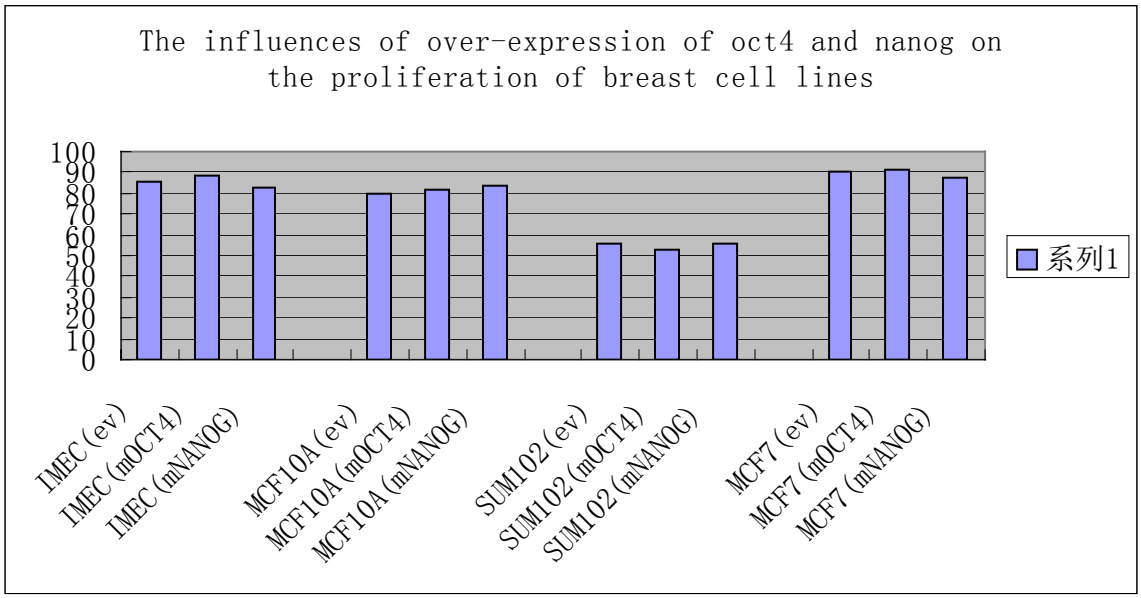
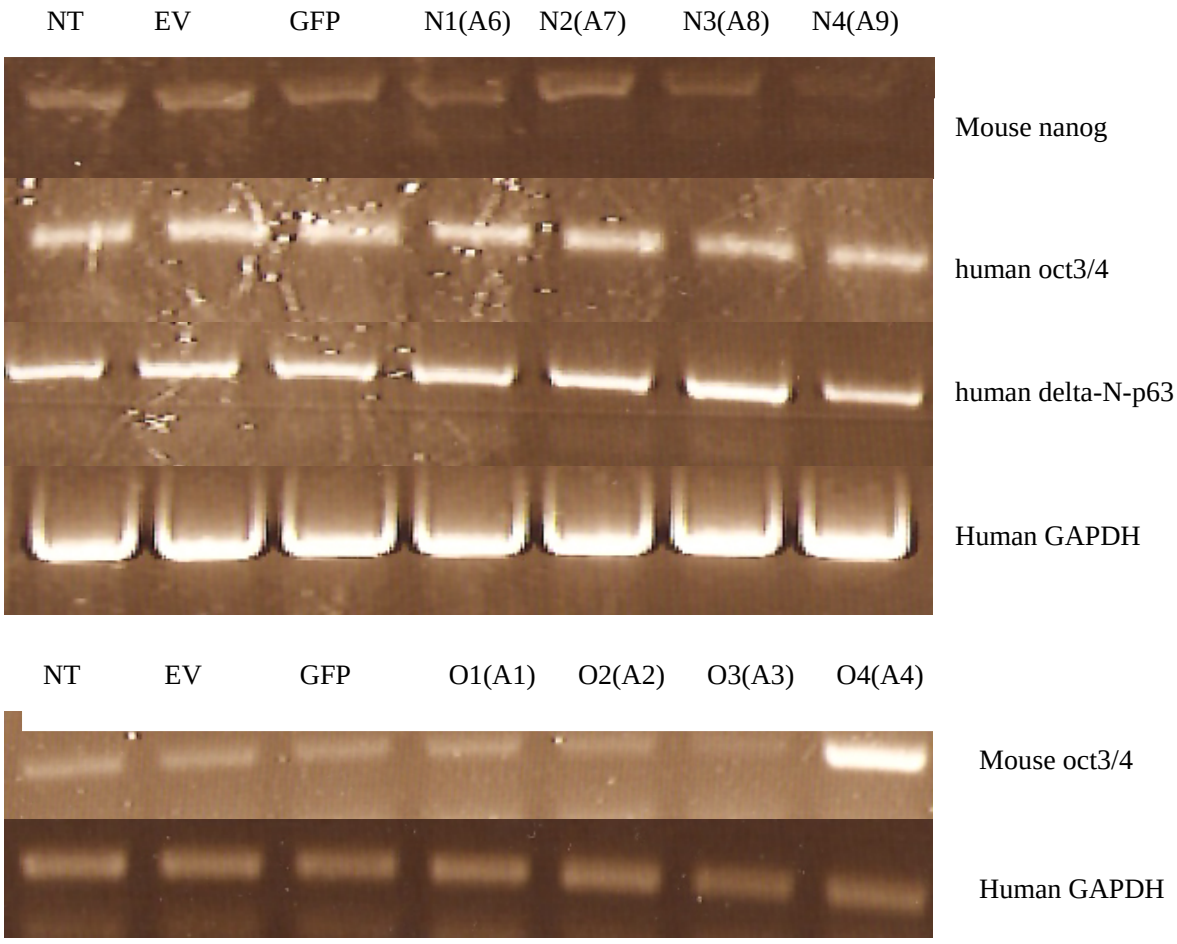

The influences of over-expression of oct4 and nanog on the proliferation of breast cell lines
100
90
80
70
60
50
40
30
20
10
0
IMEC (ev)
IMEC (mOCT4)
IMEC (mNANOG)
MCF10A (ev)
MCF10A (mOCT4)
MCF10A (mNANOG)
SUM102 (ev)
SUM102 (mOCT4)
SUM102 (mNANOG)
MCF7 (ev)
MCF7 (mOCT4)
MCF7 (mNANOG)
系列1

Figure six: Human oct3/4 and nanog knocking down with letiviruses expressiong shRNA against oct3/4 and nanog in breast cell lines

MCF-10A cells were infected with letiriruses expressing shRNA against oct3/4 and nanog, respectively. Each infection was composed of 7 groups: no treatment, empty vector control, GFP vector control for infection efficiency evaluation, other four different shRNA strains. The infected cells were harvested for cell counting directly, and the total RNA was collected for quantitative and semi-quantitative analysis.



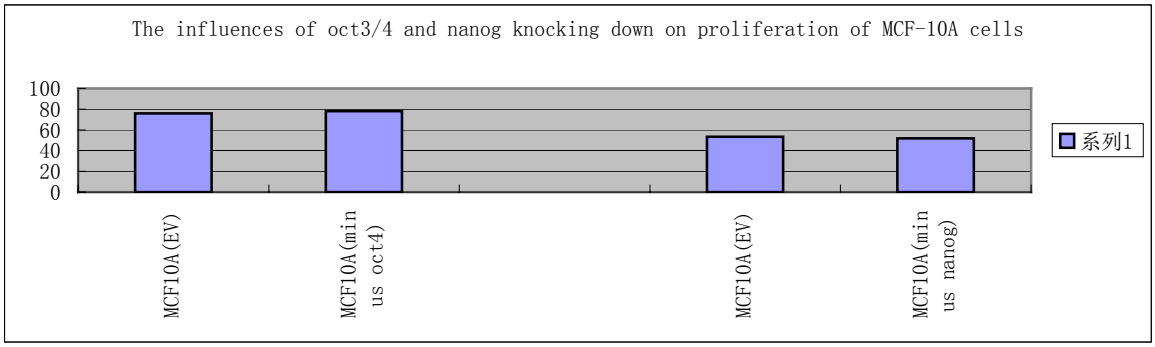
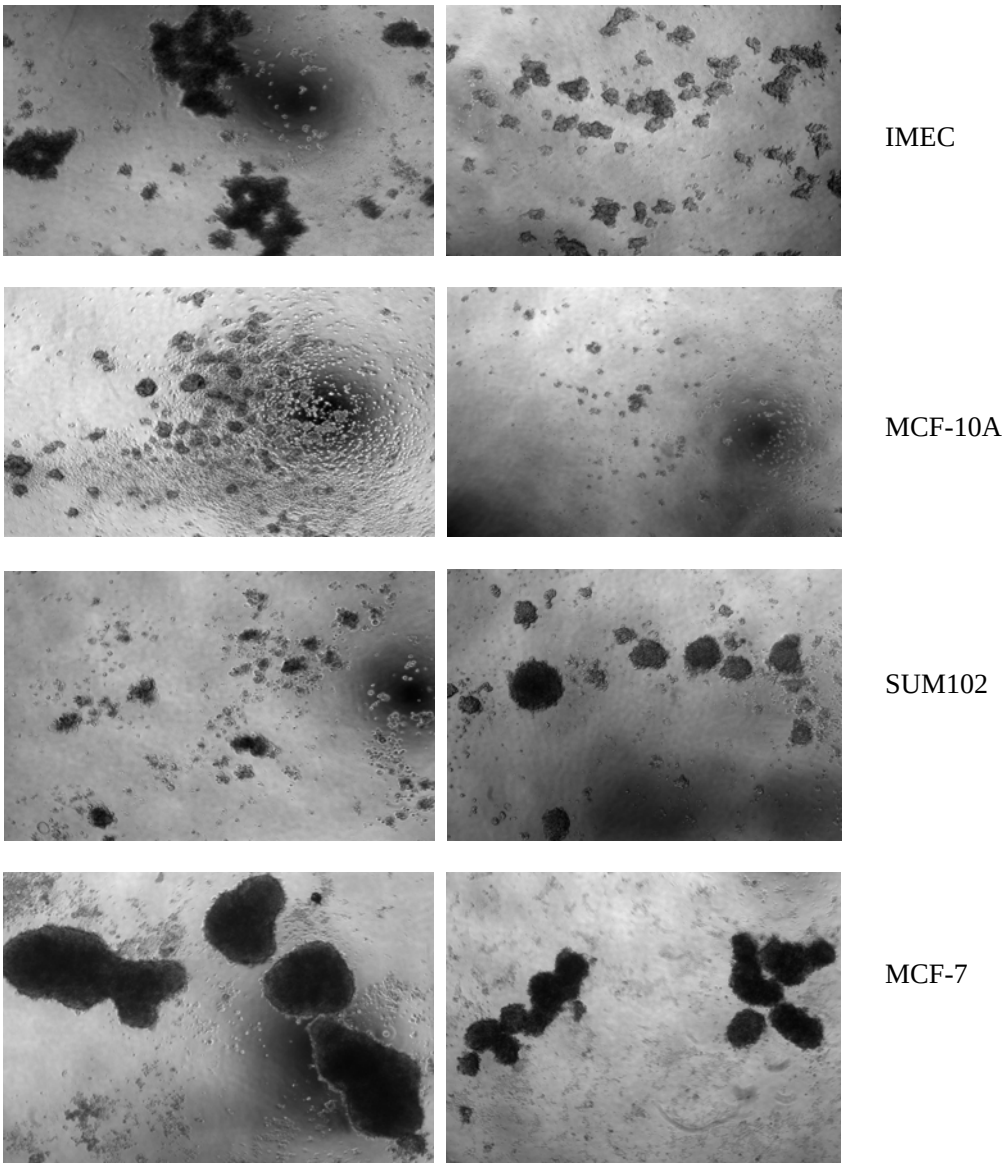


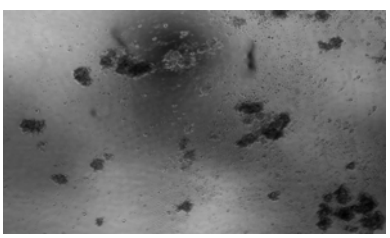
Figure seven: over-expression of ectopic human delta-N-p63, knocking down of endogenous delta-N-p63 and transfection of mouse oct3/4 and nanog, treatment of retinoic acid in breast cell lines had significant influences on formation of mammosphere.

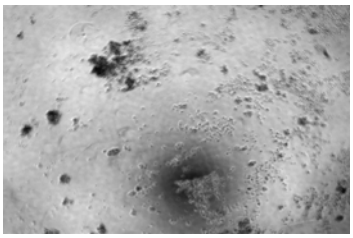
All breast cell lines were pre-treated with retinoic acid and DMSO as vehicle control, delta-N-p63 alpha and GFP empty vector adenoviruses as vehicle control, adenovirus expressing siRNA against TP63 DNA binding domain and GFP empty vector adenovirus as vehicle control, mouse oct3/4 and nanog expressing plasmid and pcDNA-3.1 as vehicle control. After treatment for 72 hours, all treated samples were harvested, then plated on 24-well low binding plates at 5×10^4/well cellular density. Primary mammosphere were formed in 10 days to 2 weeks. The mammosphere structure were collected and digested with 0.25% Trypsin and EDTA for 10 min, and re-plated on 24-well low-binding plates for secondary mammosphere formation assay.



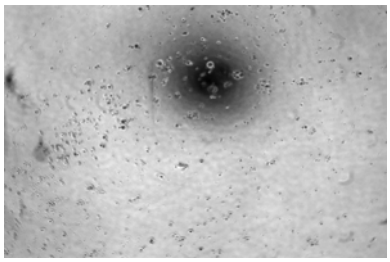
DMSO (secondary)

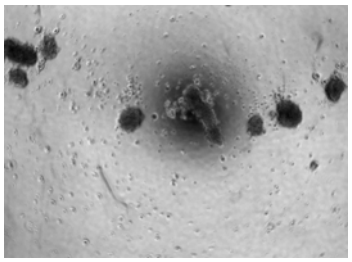
RA (secondary)



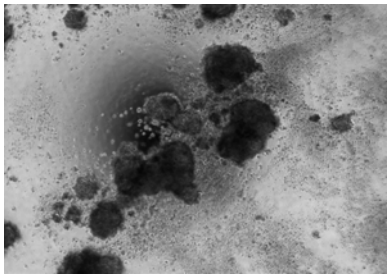


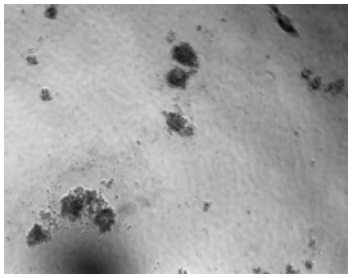
IMEC



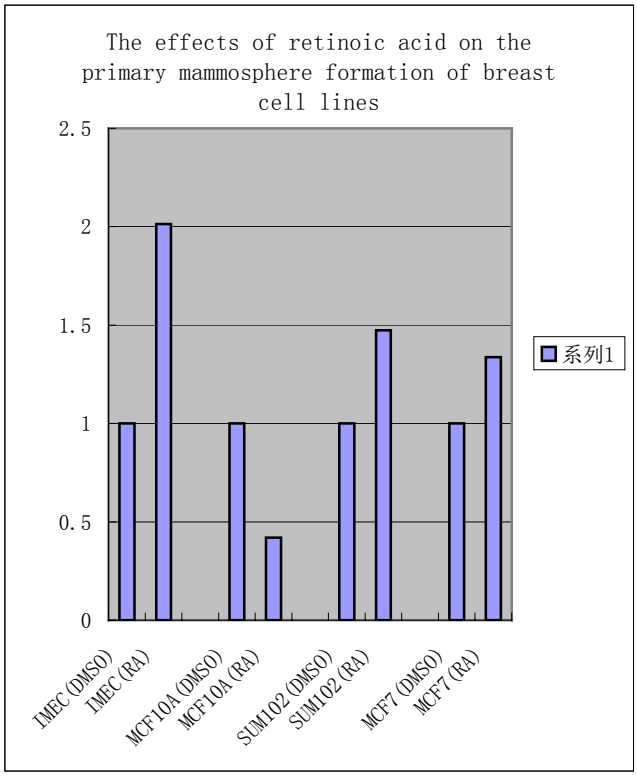


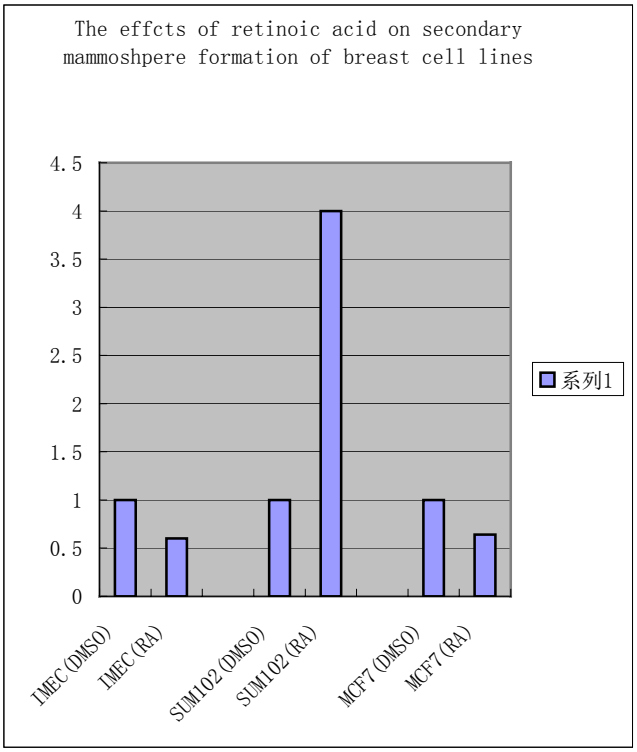
SUM102



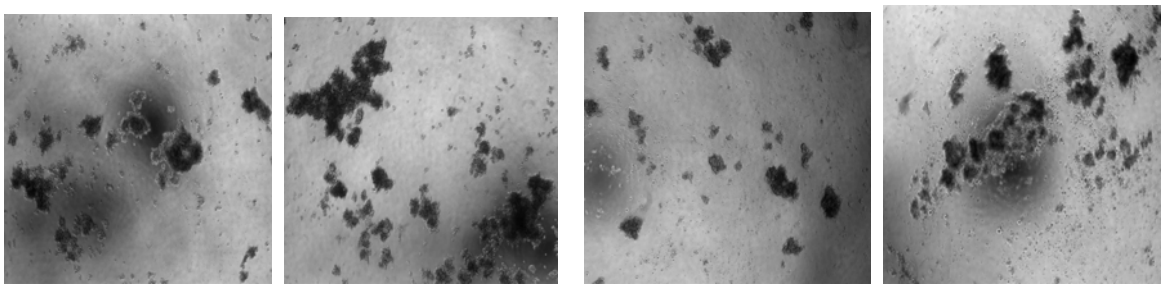


MCF-7

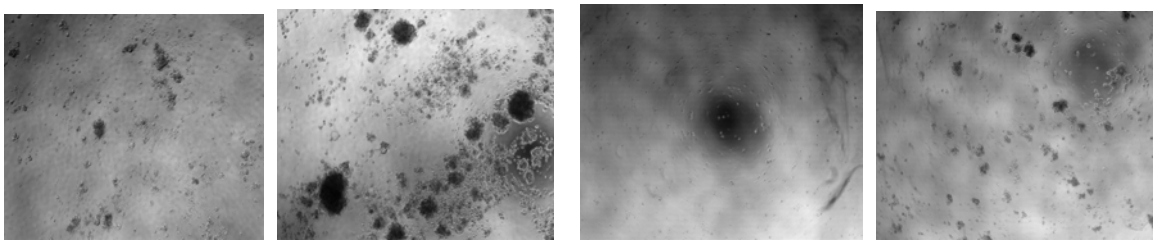




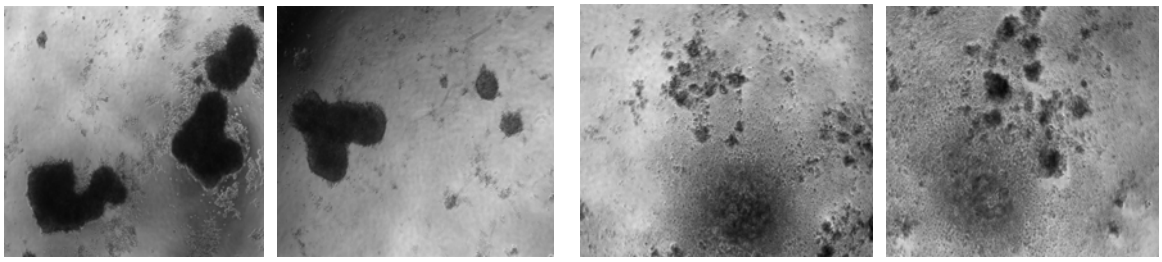
EV(primary) delta-N-p63(primary) EV(secondary) delta-N-p63(secondary)



IMEC

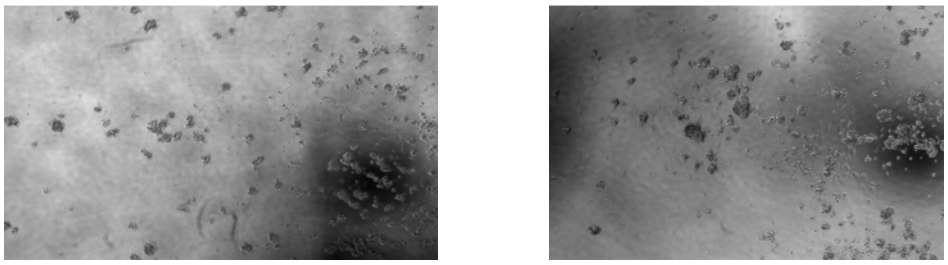


SUM102

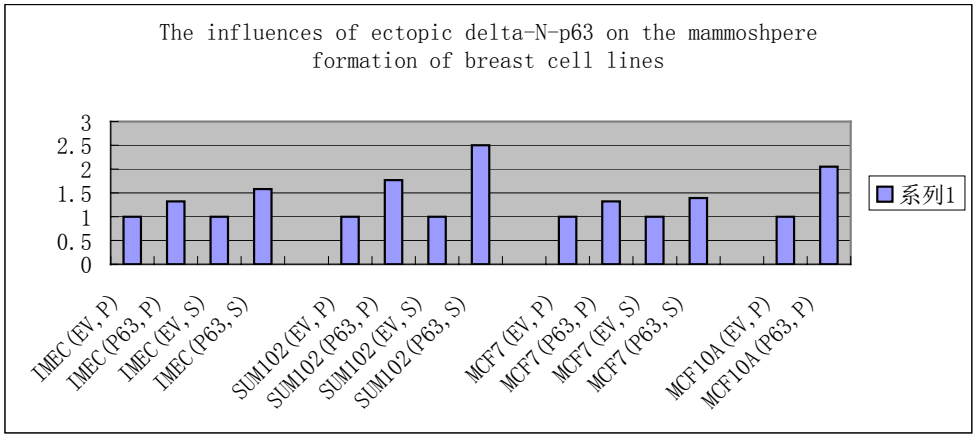


MCF-7

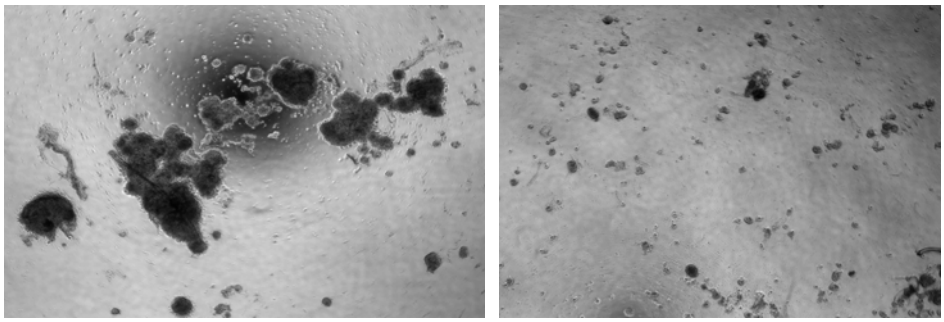
EV(primary) delta-N-p63(primary)



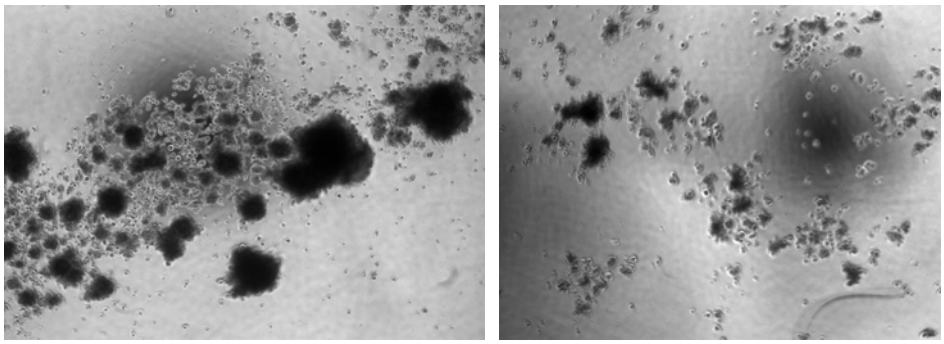
MCF-10A



EV(primary) delta-N-p63 knocking down(primary)

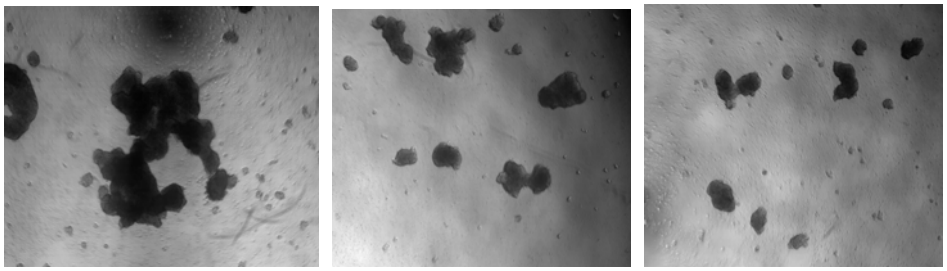


MCF10A

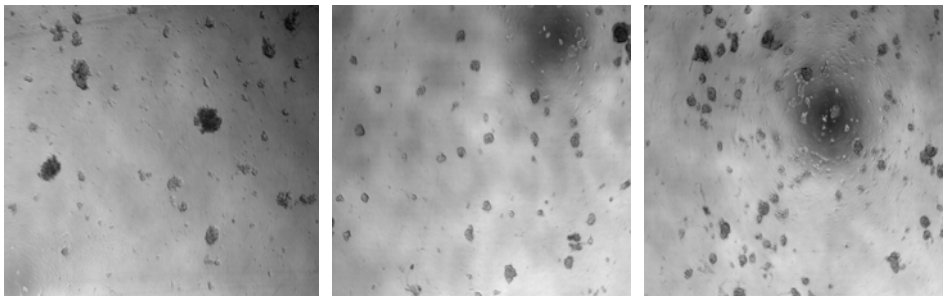


SUM102

EV mouse OCT4 mouse NANOG

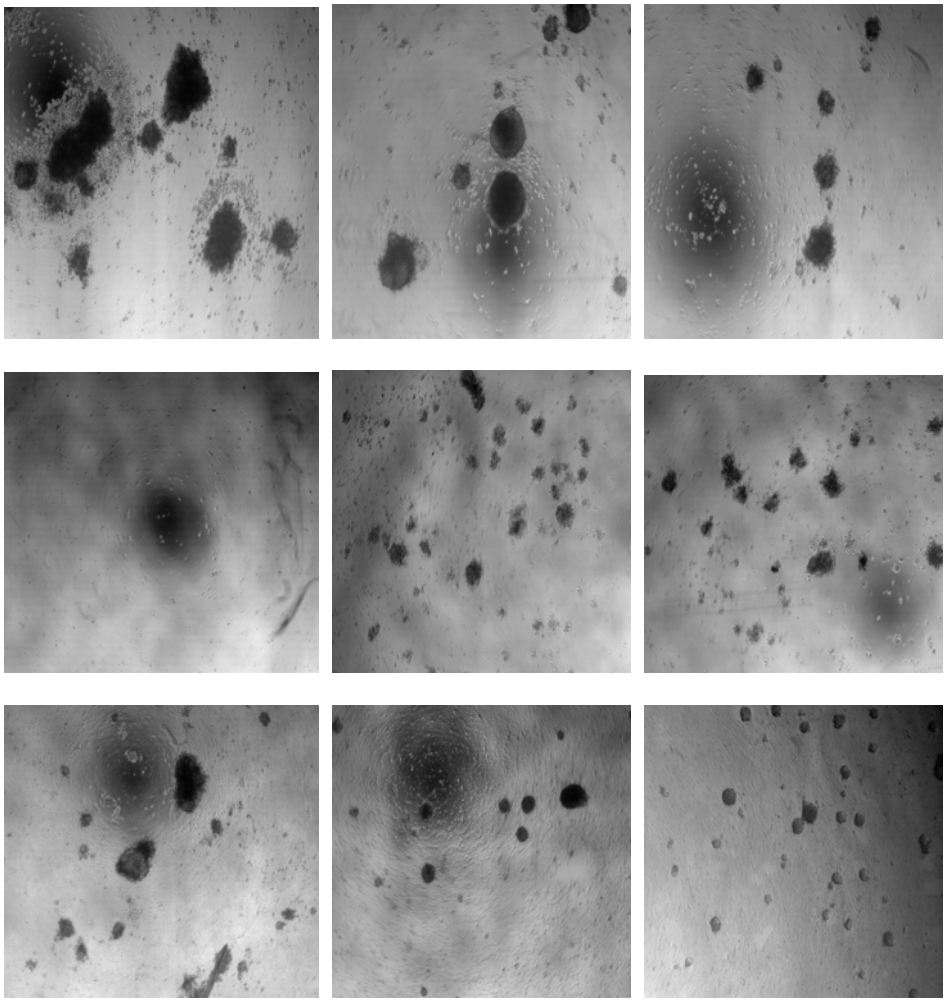


IMEC (primary)



IMEC (secondary)

EV mouse OCT4 mouse NANOG



SUM102 (primary)

SUM102 (secondary)

MCF10A (primary)

Figure eight: the transcripts of oct3/4 and nanog could be detected in mouse stem cells and progenitor cells enriched subpopulations, and delta-N-p63 could regulate their mRNA level, respetively.

Mouse stem cells and progenitor cells enriched population were isolated from wide type and PATCH (-/+) B6/129 mice at age of 10 weeks. Stem cells enriched population (P6 fraction) was treated with adenovirus expressing siRNA against TP63 DNA binding domain, progenitor cells enriched population (P5 fraction) was infected with delta-N-p63 alpha adenovirus, respectively. GFP empty vector was used as vehicle control. After infection for 72 hours, total RNA from all cell samples were harvested for semi-quantitative PCR analysis.

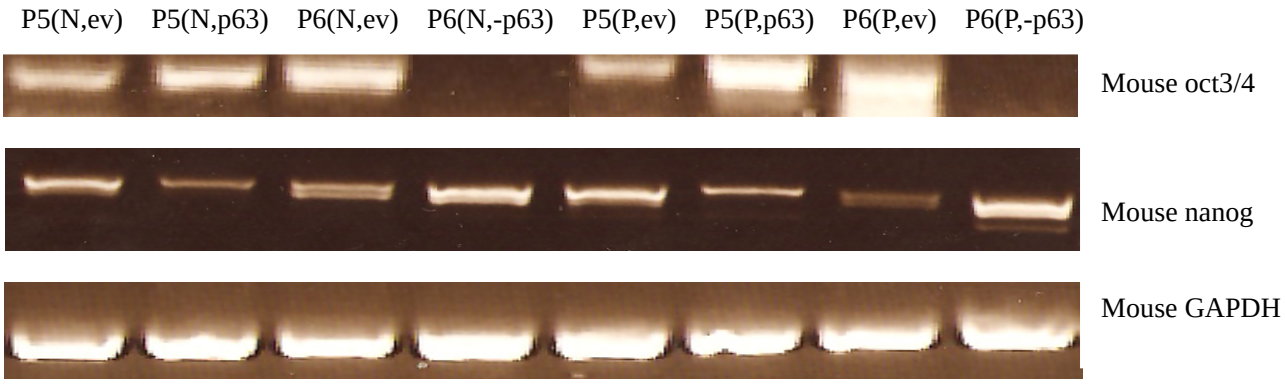
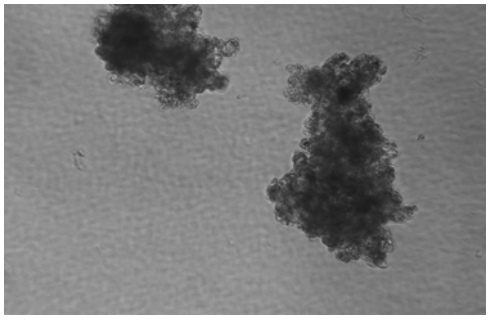
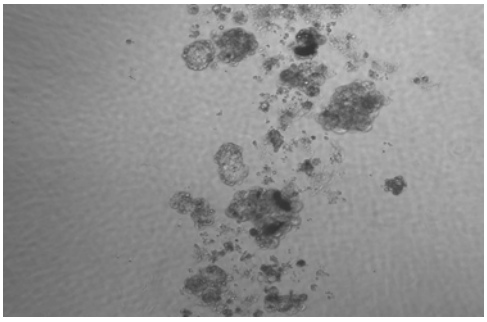


Figure nine: retinoic acid and delta-N-p63 had diverse effects on the mammosphere formation abilities of mouse mammary stem cells and progenitor cells enriched population.

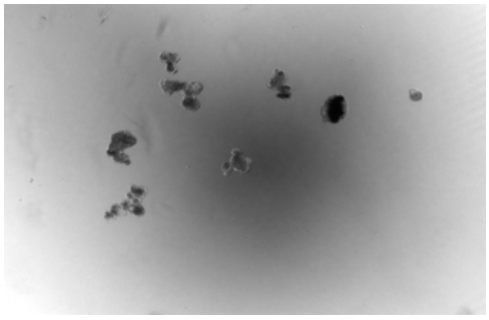
P5 (EV)



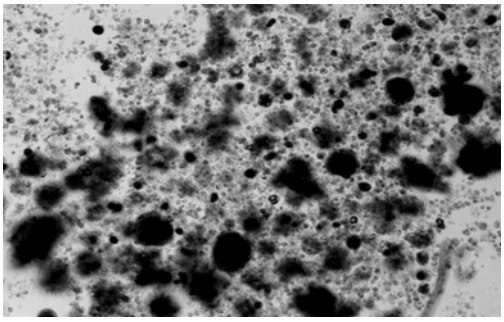
P5(P63)



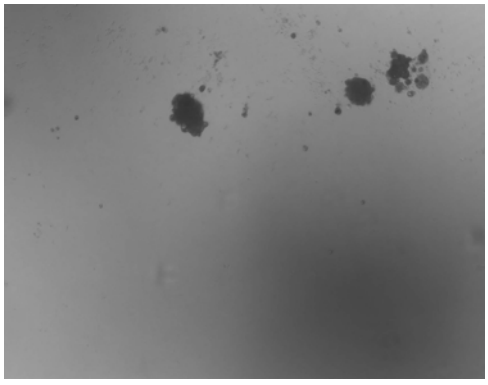
P6 (DMSO)



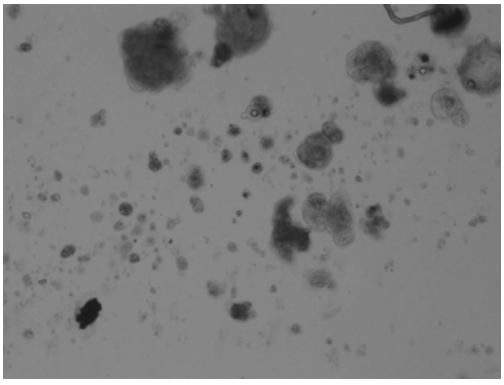
P6 (RA)



P6 (EV)



P6(minus p63)



Accomplishments:

1. Identification of nestin as a selective marker of basal/myoepithelia in the mammary gland.
2. Demonstration that expression of nestin is promoted by ΔN-p63-α.
3. Demonstration that expression of nestin positively and selectively identified the basal breast cancer subtype.
4. Demonstration of the predictive and prognostic value of nestin and ΔN-p63 in breast cancer..
5. Completion of retinoic acid treatment on various breast cell lines including IMEC, MCF-10A, SUM102 and MCF-7 cells, and further analyze its effects on proliferation rates of treated cells.
6. Quantitative and semi-quantitative PCR analysis of effects of retinoic acid on transcript level of key transcription factors in ES cells' self-renewal and multi-potential maintenance including Oct3/4, Nanog, c-myc, Klf-4 and SOX-2 in multiple breast cell lines such as IMEC, MCF-10A, SUM102 and MCF-7 cells.
7. Over-expression of ΔN-p63 mediated by ΔN-p63-α adenovirus in breast cell lines including IMEC, MCF-10A, SUM102 and MCF-7 cells, and further analyze its effects on proliferation rates of infected cells.
8. Quantitative and semi-quantitative PCR analysis of effects of ectopic ΔN-p63 on transcript level of stem cell self-renewal and pluropotency maintenance genes including Oct3/4, Nanog, c-myc, Klf-4 and SOX-2 in several breast cell lines such as IMEC, MCF-10A, SUM102 and MCF-7 cells.
9. Loss function assay of ΔN-p63 via adenoviral-shRNA positive breast cell lines such as IMEC, MCF-10A and SUM102 cells, and further investigation into the effects of removal of delta-N-p63 on their proliferation rates and transcript level of Oct3/4, Nanog, c-myc and Klf-4 in these breast cell lines.
10. Over-expression of mouse Oct3/4 and Nanog in breast cell lines including IMEC, MCF-10A, SUM102 and MCF-7 cells, analysis of effects of gain function of Oct3/4 and Nanog on cellular proliferation rates and transcript level of delta-N-p63 accordingly.
11. Knocking down of human Oct3/4 and Nanog with letivirus expression specific anti-Oct3/4 and Nanog shRNA in breast cell lines including IMEC, MCF-10A and SUM102 cells.
12. Performing mammosphere assay in mouse mammary stem cells and progenitor cells enriched epithelial subpopulations and various breast cell lines including IMEC, MCF-10A, SUM102 and MCF-7 cells, which were treated with retinoic acid, delta-N-p63 alpha adenovirus, adenoviruses expressing siRNA against delta-N-p63 alpha and TP63 DNA binding domain, respectively.
13. Western blotting of Nanog and c-myc in IMEC, MCF-10A, SUM102 and MCF-7 cells to find out the optimal antibodies for further immunohistochemsitry or immunofluorescence profiling of multiple breast cancer subtypes samples.

Reportable outcome:

N/A

Conclusions:

In this report we report on published findings related to nestin and ΔN-p63 in basal breast cancers. Additionally, in this report, we reported that existence of some transcription factors including Oct3/4, Nanog, c-myc and Klf-4, essential to maintain self-renewal and pluopotency in embryonic stem cells, in mouse mammary stem cells and progenitor cells enriched epithelial subpopulation as well as adult immortalized and malignant breast cells. Our previous study had clearly revealed the localization of ΔN-p63 in normal breast epithelial cells, mouse mammary stem cell cellular population, and selective role of ΔN-p63 in basal/myoepithelial breast cancer subtype. Taken together, this body of work indicates that the expression of ΔN-p63 in breast cancers may not only be indicative of basal epithelial subtype but may also play an important role in conferring or preserving a stem cell phenotype upon a subset of tumor cells. Additionally, retionic acid could induce cellular differentiation by down-regulation of delta-N-p63 in immortalized mammary epithelial cells (IMEC). To further investigate the biological functions of retinoic acid and ΔN-p63 in mammary cells, we analyzed the effects of retinoic acid treatments, over-expression and knock down ΔN-p63 on proliferation rates and mammosphere formation abilities in various breast cell lines. Cell counting data explicitly showed both RA treatment and over-expression of ΔN-p63 could inhibit the growth rate of treated breast cells. On the contrary, removal of delta-N-p63 itself could not slow down the proliferation rates of breast cell lines significantly. In most breast cells, over-expression of ectopic ΔN-p63 , retionic acid treatment could dramatically increase mammosphere formation capacities. Accordingly, knock down of ΔN-p63 in MCF-10A cells could decrease mammosphere numbers. Over-experssion of mouse Oct3/4 and Nanog in breast cell line such as IMCE, MCF-10, SUM102 cells had no significant effects on the proliferation rates of transfected cells, but could increase mammosphere formation ability in SUM102 cells dramatically. Quantitative and semi-quantitative PCR results explicitly demonstrated that both retinoic acid treatment and over-expression of ectopic delta-N-p63 had diverse effects on transcript levels of Oct3/4, Nanog, c-myc and Klf-4. Moreover, results of the retionic acid treatment, over-expression and removal of ΔN-p63 in mouse stem cells and progenitor cells subpopulation also revealed the key role of ΔN-p63 in self-renewal and multi-potential maintenance regulation process in adult mammary gland.

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